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Appendix C

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Isolation and Characterization of a Conserved Porin Protein from *Helicobacter pylori*

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***Helicobacter pylori* is a causative agent of gastritis in humans and is correlated with gastric ulcer formation. Infections with this bacterium have proven difficult to treat with antimicrobial agents. To better understand how this bacterium transports compounds such as antimicrobial agents across its outer membrane, identification of porin proteins is important. We have recently identified a family of *H. pylori* porins (HopA to HopD) (M. M. Exner, P. Doig, T. J. Trust, and R. E. W. Hancock, Infect. Immun. 63:1567–1572, 1995). Here, we report on an unrelated porin species (HopE) from this bacterium. This protein had a apparent molecular mass of 31 kDa and was seen to form 50- and 90-kDa aggregates that were designated putative dimeric and trimeric forms, respectively. The protein was purified to homogeneity and, with a model planar lipid membrane system, was shown to act as a nonselective pore with a single channel conductance in 1.0 M KCl of 1.5 nS, similarly to other bacterial nonspecific porins. An internal peptide sequence of HopE shared homology with the P2 porin of *Haemophilus influenzae*. HopE was also shown to be antigenic in vivo as assessed by sera taken from *H. pylori*-infected individuals and was immunologically conserved with both patient sera and specific monoclonal antibodies. From these data, it appears that HopE is a major nonselective porin of *H. pylori*. The implications of these findings are discussed.**

Helicobacter pylori is a gram-negative bacterium that ranges in shape from curved to spiral and colonizes the stomachs of humans (11, 23, 34). Once acquired, *H. pylori* persists within its host for years, possibly for life, if left untreated (5). Epidemiological studies have consistently demonstrated an association between *H. pylori* infection and type B gastritis, peptic ulceration, and the development of intestine-type gastric adenocarcinoma (6). Most recently, *H. pylori* has been recognized as a risk factor for primary B-cell gastric mucosa-associated lymphoid tissue lymphoma (17).

The significant pathologies attributed to infection by this organism have led to a desire to eradicate the bacterium by antibiotic therapies. However, *H. pylori* has proven difficult to treat, with in vivo susceptibilities being poorly correlated to those determined in vitro (12, 25, 30). Indeed, an aggressive regime involving triple drug therapy is frequently required (39). Such aggressive drug therapies are expensive, are fraught with potential patient side effects, and are not always successful. Thus, a better understanding of the factors that determine the susceptibility of *H. pylori* to antimicrobial agents would lead to more-effective and less-aggressive treatment regimens.

In vivo susceptibility to antibiotics is influenced by a number of factors, both host and pathogen dependent. A major factor influencing in vivo antimicrobial susceptibility is the permeability of the bacterium to a given antimicrobial agent (14, 28). Also confounding treatment for *H. pylori* is the fact that many antimicrobial agents have been selected in part on the basis of their rapid absorption and hence are quickly removed from this bacterium's gastric mucosal niche. Therefore, ease of permeability of an antimicrobial agent into *H. pylori* has added significance in determining susceptibility.

In gram-negative bacteria, the outer membrane acts as a selective barrier, determining what may enter the cell. Small hydrophilic molecules can cross the hydrophobic outer membrane by using small hydrophilic pores, which are composed of proteins termed porins, inserted in the outer membrane (1, 14). Indeed, β-lactams, as well as many small hydrophilic antimicrobial agents, primarily use porin channels to cross the outer membrane (14, 28).

Despite the possible significance of porins in determining the susceptibility of a bacterium to antimicrobial agents, little is known about this important class of proteins from *H. pylori*. Recently, Tufano et al. (36) have proposed that a 30-kDa outer membrane protein may be a porin based on gel migration properties. However, it has not yet been determined whether this molecule can form channels in lipid bilayers and, if so, what are the properties of these pores in participating in the permeability of the *H. pylori* outer membrane. In this study, we have purified an outer membrane protein with an apparent molecular mass of 31 kDa (henceforth termed HopE) and determined that this protein acts as hydrophilic pore with a channel size similar to that of other gram-negative porins. This porin likely represents the major porin species of this bacterium and may be analogous to the protein reported by Tufano et al. (36), which possesses immunomodulatory activity.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *H. pylori* strains examined in this study were the endoscopic biopsy isolates CCUG 17874 (identical to the type strain NCTC 11637, isolated by B. J. Marshall at Royal Perth Hospital, May 1982 [26]) and 5155 (isolates from Sydney, Australia) and strain A5 (Swedish isolate, Astra Arcus AB, Södertalje, Sweden). Cultures of *H. pylori* were grown at 37°C on chocolate blood agar plates in an atmosphere containing 10% (vol/vol) carbon dioxide for 48 h.

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Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method described by Laemmli (22) with a mini-slab apparatus. Protein was stained with Coomassie blue R-250. When required, separated proteins were transferred from the polyacrylamide slab gel to a nitrocellulose membrane according to the method described by Bjerrum and Schafer-Neilsen (4). Electroblotting was carried out in a semidry transblot apparatus (LKB, Baie d'Urfe, Quebec, Canada) for 30 min at a field strength of approximately 0.8 mA/cm^2. Western blots (immunoblots) were blocked, incubated, and developed as described previously (7).

Human sera. Human sera were obtained from *H. pylori*-infected patients being treated at the Vancouver General Hospital (Vancouver, British Columbia, Canada). The patients were assessed positive by biopsies. Control sera were taken from apparently uninfected individuals (n = 4).

Purification of functional HopE protein. *H. pylori* CCUG 17874 was grown on chocolate blood agar for 48 h in an environment of 10% CO_2. Cells were harvested in 10 mM Tris (pH 7.5), and outer membranes were prepared by the Triton X-114 extraction method as described previously (7). The membrane preparation was mixed with an equal volume of 0.125 M Tris (pH 6.8)–4% (wt/vol) SDS–20% (vol/vol) glycerol–10% (vol/vol) 2-mercaptoethanol, and the proteins were then separated by SDS-PAGE (12.5% total acrylamide). The segment of the unstained gel in which HopE migrated (assessed by a Western immunoblot by using a monoclonal antibody [MAb] specific for this antigen) was cut out, and the protein was eluted from the gel slice by suspension in water overnight at 4°C. The sample was concentrated by using a 10-kDa-cutoff microconcentrator (Filtron Technology Corp., Northborough, Mass.). The purified protein was obtained after one further round of SDS-PAGE and elution. The purity of the sample was assessed by SDS-PAGE, and its identity confirmed by Western immunoblotting with a HopE-specific MAb (7).

Characterization of pore-forming ability of the HopE protein. The pore-forming ability of the purified HopE protein was assessed in a model membrane system by using planar lipid bilayers (2). For single-channel conductance measurements, lipid bilayers made from 1.5% (wt/vol) oxidized cholesterol in n-decane were formed across a 0.2-mm^2 hole separating two compartments of a Teflon chamber containing a variety of salt solutions. Calomel electrodes were implanted into each compartment; one was connected to a voltage source, and the other was connected to a current amplifier, oscilloscope, and chart recorder. Approximately 10 ng of purified protein, solubilized in 0.1% Triton X-100, was added to one compartment, and a voltage of 50 mV was applied across the lipid bilayer. Increases in conductance were recorded, and average single-channel conductance was calculated for the HopE protein in each of the salt solutions used.

Zero current membrane potential experiments in Teflon chambers containing 0.1 M KCl (6.0 ml per side) were performed. The compartments of each chamber were separated by a hole larger (2.0 mm^2) than that used for the single-channel conductance measurements. A voltage of 20 mV was applied, and approximately 20 ng of protein was added. The conductance was allowed to increase for approximately 10 min until it reached 2.5×10^{-10} A, and then the voltage was removed. A 100-μl aliquot of 3.0 M KCl was added to one side of the membrane, while 100 μl of 0.1 M KCl was added to the other. Eight additions were made, and the 0 current potential was measured after each addition.

Cross-linking studies. Cross-linking was performed by using the amino-group-specific cross-linkers dithiobis(succinimidylpropionate) (DSP) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). For EDC cross-linking, outer membrane or purified HopE was suspended in 10 mM phosphate-buffered saline (pH 7.5), and EDC was added to the appropriate final concentration from a EDC stock solution. The reaction was allowed to proceed for 30 min on ice. The cross-linking reaction was stopped by the addition of Tris buffer (pH 7.5) to a final concentration of 0.1 M.

For DSP cross-linking, the cross-linker was added to outer membrane or purified HopE from a stock solution dissolved in dimethyl sulfoxide. All samples were adjusted such that they contained 10% (vol/vol) dimethyl sulfoxide. After 5 min at room temperature, the reaction was stopped by the addition of Tris buffer (pH 7.5) to a final concentration of 0.1 M.

The state of aggregation of HopE was then assessed by SDS-PAGE and Western blotting with MAb 4B9 as a probe. In the case of DSP cross-linked samples, aliquots were prepared in SDS-PAGE sample buffer with and without the addition of 2-mercaptoethanol.

N-terminal sequencing. Digestion of the HopE protein with endoproteinase Glu-C was performed as follows. Purified HopE was mixed with an equal volume of SDS-PAGE solubilization buffer, and the mixture was heated at 100°C for 10 min. To this solution, endoproteinase Glu-C was added to a final ratio of 1:200 (milligrams of protease to milligrams of HopE protein), and the mixture was incubated for 1 h at room temperature. This preparation was then subjected to SDS-PAGE. The separated peptides were transferred to a polyvinylidene difluoride membrane and stained with Coomassie blue, and the N-terminal amino acid sequence was determined as described previously (21).

Epitope mapping of HopE. Partial proteolytic digests of HopE were generated with strains CCUG 17874, 5155, and A5. Envelopes prepared as described previously (7) were suspended in 1% (wt/vol) SDS–10 mM Tris (pH 7.5), and the suspensions were heated at 100°C for 10 min. After the sample had been cooled to room temperature, chymotrypsin was added to a final concentration of 3 μg/ml. The samples were then mixed with an equal volume of SDS-PAGE sample buffer, and the mixtures were heated at 100°C for 10 min. Peptides separated by SDS-PAGE were transferred to nitrocellulose membranes and were developed by using MAbs 1G1, 4B9, and 6H2 as described previously (7).

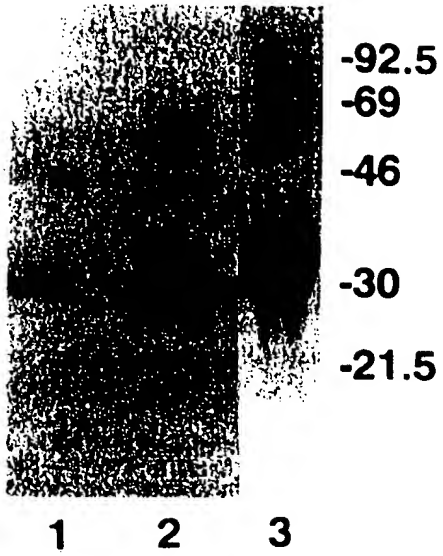

FIG. 1. Demonstration of heat modifiability and purity of HopE from *H. pylori* CCUG 17874. Lanes: 1 and 2, Western immunoblots of cell envelopes probed with MAb 4B9 (lane 1 was not heated prior to SDS-PAGE, whereas the sample in lane 2 was heated at 100°C for 15 min); 3, Coomassie blue-stained SDS-polyacrylamide gel of purified HopE. Molecular mass markers (in kilodaltons) are given on the right.

RESULTS

Purification of HopE. HopE exhibited heat-modifiable migration on SDS-polyacrylamide gels. Heating the protein for 15 min at 100°C resulted in an apparent molecular mass shift from 28 to 31 kDa, as determined by SDS-PAGE (Fig. 1, lanes 1 and 2). This migration pattern led us to believe that this protein could be an outer membrane porin; thus, purification of the native form of this protein was undertaken.

HopE was purified to homogeneity (Fig. 1, lane 3). This was achieved by repeated cycles of cutting the protein from an SDS-polyacrylamide gel. The purity of this protein was assessed by SDS-PAGE, and its identity was confirmed by Western immunoblotting with a MAb (4B9) specific for HopE. This preparation was then tested for pore-forming ability in a lipid bilayer model system.

Assessment of pore-forming ability. Purified HopE exhibited pore-forming activity in a planar lipid bilayer model membrane system. Only upon the addition of approximately 10 ng of HopE were stepwise conductance increases observed, indicating insertions of the pore-forming protein molecules into the lipid bilayer (Fig. 2). Single-channel conductance observed for each salt solution appeared to be distributed around a single mean (Fig. 3), and the average single-channel conductance for HopE in 1.0 M KCl was 1.5 nS (Table 1). Single-channel conductance was found to be a linear function of the salt concentration, and this indicated that HopE was a nonspecific, large, water-filled channel. Experiments with a larger anion (CH_3COO^-) and a larger cation (Li^+) showed little variation in conductance, which suggested only minimal selectivity for anions or cations. This was confirmed by determining the 0 current potential and by calculating the permeability ratio for anions and cations. This ratio for HopE, i.e., Pc/Pa, where Pc is the permeability for cations and Pa is the perme-

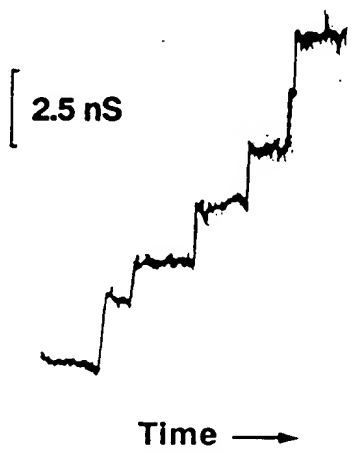

FIG. 2. Membrane conductance increase after the addition of purified HopE to a model membrane system. Each step represents the insertion of a single pore into the membrane and its resulting increase in conductance across the membrane.

ability for anions as calculated by the Goldman-Hodgkin-Katz equation, was 1.059 ± 0.001.

Cross-linking of HopE. When the effect of DSP cross-linking on HopE present in outer membranes was analyzed, MAb 4B9-reactive bands with apparent molecular masses of 50, 76, and 87 kDa were visible (Fig. 4a). These were assigned the identities of dimeric (50-kDa) and trimeric (76- and 87-kDa) forms of HopE, respectively. Furthermore, the monomeric form of HopE ran as a dimer with apparent molecular masses of 31 and 28 kDa. Similar results were obtained with the cross-linker EDC (8). Treatment of the DSP cross-linked preparations with 2-mercaptoethanol prior to SDS-PAGE resulted in the loss of the 87-, 76-, 50-, and 28-kDa bands (Fig. 4a).

When cross-linking of purified HopE was attempted, negligible subunit aggregation was observed with DSP (Fig. 4b) or EDC (8). Only a very faint band with an apparent molecular mass of 50 kDa was observed at the highest concentrations of cross-linker employed.

N-terminal analysis of HopE. Repeated attempts at N-terminal sequencing with different preparations of HopE were unsuccessful. However, peptide fragments of this protein were generated by endoproteinase Glu-C, and an approximately 20-kDa fragment was sequenced (Fig. 5). This peptide had sequence homology to the P2 outer membrane porin of *Haemophilus influenzae* (9), with 41% identity with 55% identical or conserved residues as determined by a Blastp search with GenBank. Residues 3 through 17 of the 20-kDa peptide had 71% identical or conserved residues compared with the corresponding P2 porin amino acid residues. Another interesting feature of the derived sequence was an amino acid at position 28 that had an aberrant retention time, indicating that this residue might in some way be modified.

TABLE 1. Average single-channel conductance of the *H. pylori* HopE porin in a lipid bilayer model system under varying physical conditions

Salt (concn)	*n*[a]	λ (nS)[b]	σ (mS/cm)[c]
KCl (0.3 M)	88	0.45	34
KCl (1.0 M)	129	1.5	112
KCl (3.0 M)	90	2.95	250
LiCl (1.0 M)	89	0.86	71
CH_3COOK (1.0 M)	103	0.65	68

[a] *n*, number of measured events.
[b] λ, conductance.
[c] σ, conductance.

Antigenicity of HopE. Six patients infected with *H. pylori* (assessed by positive biopsy) were screened for serum antibody to the purified HopE protein. All *H. pylori*-positive patients' sera recognized this protein by Western immunoblots (Fig. 6).

To further investigate the immunological diversity of HopE, chymotrypsin-generated peptides were obtained from three

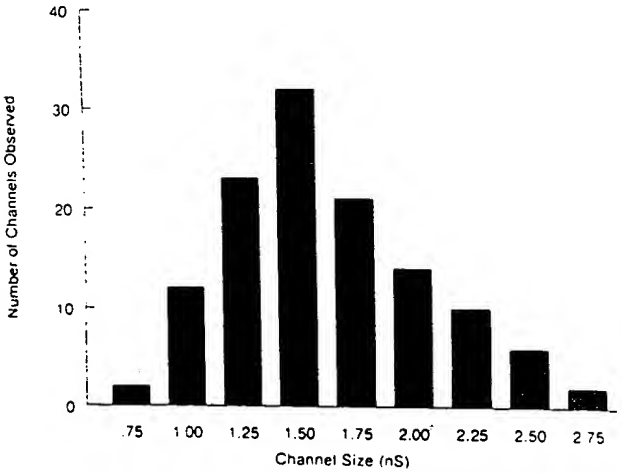

FIG. 3. Frequency histogram of conductance for HopE measured in a model membrane system with 1.0 M KCl (pH 7.0) as an electrolyte. The frequency of insertion of each sized pore (as determined by the increase in the magnitude of conductance) was charted to determine the average single conductance and demonstrated a distribution around a single mean.

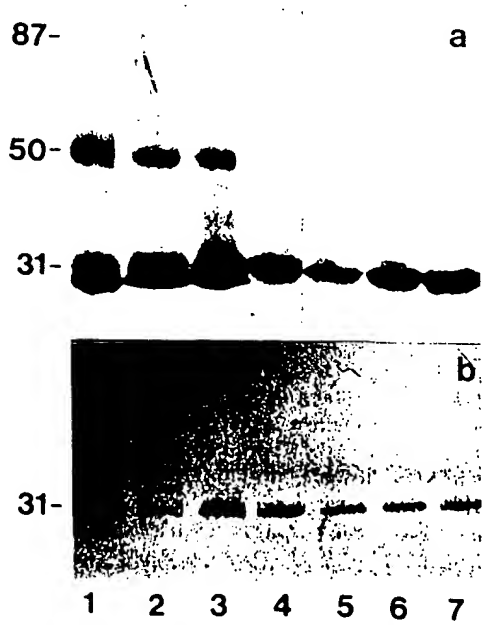

FIG. 4. Western immunoblot showing the effect of the cross-linker DSP on the aggregation state of HopE. (a and b) Samples treated with the following concentrations of DSP: 200 μg/ml (lanes 1 and 5), 100 μg/ml (lanes 2 and 6), 50 μg/ml (lanes 3 and 7), and 4.0 μg/ml (lane 4). Lanes 1 through 3 were heated in SDS-PAGE sample buffer without 2-mercaptoethanol, while lanes 4 through 7 were heated in SDS-PAGE sample buffer containing 0.5% 2-mercaptoethanol. Apparent molecular masses are given on the left (in kilodaltons).

Protein	Sequence[a]
HopE peptide	EGDGVYIGTNYQLGQARLNSNIYNTGDXTG
	||: :| || | ||| :| |:
H. influenzae P2 porin	GIDGLVLGANYLLAQARDTANPGKQGEVAA

FIG. 5. Homology of an N-terminal sequence of a 20-kDa endoproteinase Glu-C-generated peptide derived from HopE with the *H. influenzae* P2 porin. The X at residue 28 represents a undetermined modified amino acid. Vertical lines, identical residues; cola, conserved substitutions. The *H. influenzae* P2 porin sequence is from Duim et al. (9).

strains of *H. pylori* isolated from diverse geographic regions. These three strains also had HopE monomers with differing molecular masses as determined by SDS-PAGE (CCUG 17874, 31, 32, and 34 kDa, respectively). With three MAbs specific for HopE, strains CCUG 17874 and A5 showed extremely similar peptide fingerprints (Fig. 7). While chymotrypsin-digested HopE from strain 5155 produced some peptides identical in apparent molecular mass to those of A5 and CCUG 17874, two major peptides were significantly different (Fig. 7).

DISCUSSION

The outer membrane of gram-negative bacteria is a primary barrier to hydrophilic molecules entering the cell (14). Transport across this barrier is facilitated by porin proteins which form channels through which hydrophilic solutes may travel. These proteins may transport specific metabolites, or they may be nonspecific, allowing hydrophilic molecules smaller than their pore size to enter. In the case of *H. pylori*, a family of related porin species (termed HopA through HopD) has been described elsewhere (10). This group of porins was classed together on the basis of shared N-terminal amino acid residue homology. They are characterized by low single-channel conductance by model membrane techniques, as well as by their relatively low relative abundance in the outer membrane compared with major porins from other bacterial species.

Monomer porins and low-single-channel conductance are typical of porins isolated from bacterial species related to *H. pylori*, such as *Campylobacter coli* (0.53 nS) (18), *Campylobacter jejuni* (0.82 nS) (29), and *Campylobacter rectus* (0.49 and 0.60) (19). Nonetheless, it seemed possible that *H. pylori* possesses another class or species of porin(s) that might resemble the major porins of other gram-negative bacteria. Tufano et al. (36) have described a protein produced by *H. pylori* with a molecular mass of 30 kDa that was presumed to be a porin on the basis of its heat-modifiable behavior on SDS-PAGE, although pore-forming ability was not examined. In this study, we have purified a protein with an apparent molecular mass of 31 kDa (HopE) from the outer membrane of *H. pylori* and determined that it functions as a porin. Attempts to purify the trimeric form of the HopE were unsuccessful because of its instability in SDS; however, the monomeric form was purified

FIG. 6. Immunoreactivity of human sera with purified HopE assessed by Western immunoblotting. Lanes 1 through 6 were incubated with sera for *H. pylori*-infected patients, whereas lane 7 was serum from an uninfected subject.

FIG. 7. Peptic immunofingerprints of HopE partially digested with chymotrypsin. Blots were incubated with 1G1 (a), 6H2 (b), and 4B9 (c). The blots shown in panels a and b were loaded with chymotrypsin-digested HopE from CCUG 17874 (lane 1), 5155 (lane 2), and A5 (lane 3). The blot shown in panel c was loaded with chymotrypsin-digested HopE from CCUG 17874 (lane 1), A5 (lane 2), and 5155 (lane 3) Molecular mass markers (in kilodaltons) are given on the left.

to homogeneity, and it formed functional channels in lipid bilayers. The instability in SDS of the HopE trimer is not an uncommon feature of porins trimers, since it is reported for a number of other bacterial species (18, 20, 29, 37, 38). Cross-linking experiments indicated that the HopE protein forms trimers in the outer membrane, indicating that HopE likely functions as a trimer in vivo. Despite differences in SDS stability and molecular masses, which might be explained by strain variation, the HopE reported here and the presumptive porin of Tufano et al. (36) are likely the same or related protein(s).

Trimer formation was not noted in the purified HopE preparation when cross-linking experiments were performed. Therefore, it is possible that the pore conductance measurements reported here are for the monomeric form of HopE rather than the trimeric form. The single-channel conductance measurements observed for apparent HopE monomers indicated that it formed large channels compared with other porins so far described for *H. pylori*. It is also large compared with channels typically seen in other gram-negative bacteria. For instance, the single-channel conductance of the HopE trimer would be approximately 4.5 nS in 1.0 M KCl, whereas OmpC, OmpF, and PhoE from *Escherichia coli*, and OmpC and OmpF from *Salmonella typhimurium* have single-channel conductance values of between 1.5 and 2.4 nS (3). Of course, the protein could have been reconstituting into the lipid bilayer as trimer units, in which case its single channel conductance would be within the range of the porins described above. Porins with large channel sizes usually allow passage of hydrophilic substances by general diffusion, and this is likely the case with HopE. The conductance measurements showed a linear relationship between salt concentration and conductance, indicating that a specific binding site is not present in the pore (in which case conductance would saturate with increasing concentration). Furthermore, 0 current membrane potential results showed virtually no selection for cations over anions, which again reinforced the assumption that HopE is a general diffusion pore. Nonspecific porins are frequently involved in the transport of hydrophilic antimicrobial agents across the outer membrane (14). Thus, because of its large pore size and nonspecific nature, HopE should play a significant role in the transport of antimicrobial agents across the outer membrane of *H. pylori*. Since this protein is present in the outer membrane at a relative level of abundance lower than those of many major porins from other bacteria, this may partially contribute to antibiotic resistance. However, this must be qualified since we do not know the level of HopE expression in vivo.

An interesting property of HopE, and one shared with members of the other class of porins from *H. pylori*, is that it was not

peptidoglycan associated (7), as determined by the method described by Rosenbusch (31). Virtually all porins from gram-negative bacteria that have been characterized to date have shown association with the peptidoglycan. This includes porins from various *Campylobacter* species. The reason for the apparent lack of peptidoglycan association is not known. However, it is possible that the porins of *H. pylori* still interact with the peptidoglycan, but by a novel form of association compared with other bacterial porins. For example, OprP, the phosphate-specific porin of *Pseudomonas aeruginosa* can be simply removed from the peptidoglycan by EDTA treatment, even in very mild detergents (15).

Despite the inability to determine the N-terminal amino acid sequence of HopE, an internal endoproteinase Glu-C proteolytic peptide was successfully sequenced. This peptide exhibited some homology to the P2 porin of *H. influenzae* (residues 162 to 186). Residues 3 through 17 had the greatest homology and correspond to a membrane-spanning region of P2 that is thought to be involved in pore channel formation (33). Residues 18 through 30 were less conserved. This region corresponds to a hypervariable surface-exposed region within P2 (residues 177 to 188) (33).

By using MAbs, HopE was shown previously to be highly conserved serologically among *H. pylori* strains, but not among other *Helicobacter* spp. (7). However, these MAbs did cross-react with a presumptive porin in *C. coli*. More extensive screening of *H. pylori* isolates with these MAbs has further confirmed the conservation of this protein immunologically (8). Virtually identical proteolytic immunofingerprints were recognized by these three MAbs to HopE, indicating that they are specific for similar or identical epitopes on the protein. With respect to strain-to-strain variation of HopE, two of the three strains examined had identical proteolytic immunofingerprints. The other strain examined varied with respect to one major peptide band. Furthermore, some apparent molecular mass variation was observed in different strains, with a range of 29 to 34 kDa being noted (8). These variations indicate that differences in the primary amino acid sequence of HopE between strains occurs, which is a feature not uncommonly found among porins (9, 24, 33).

Porins, including the P2 porin of *H. influenzae*, have been used as immunogens that are actively and/or passively protective in subsequent challenge experiments (13, 16, 27, 32). However, serotypic variation of these porins has limited their usefulness as vaccine candidates (13, 16, 35). Unlike HopA, -B, -C, and -D *H. pylori* porins, HopE appears to be serotypically conserved. Tufano et al. (36) has previously reported that a 30-kDa heat-modifiable protein from *H. pylori* was able to stimulate cytokines. Here, we have demonstrated positive serology of patients infected with *H. pylori* to HopE from a single strain, further demonstrating its immunogenicity and immunoconservation.

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